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PRINCIPAL INVESTIGATOR: Peter O'Connell, Ph.D.

CONTRACTING ORGANIZATION: Baylor College of Medicine
Houston, Texas 77030

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13. ABSTRACT (Maximum 200 Words) Some breast cancers spread (metastasize) to distant sites, putting the patient at high risk of death from this disorder. Clinicians now use tumor size, tumor appearance, and especially the presence of metastasis (cancer spread to local lymph nodes, or "node-positive breast cancer") to estimate the risk of early breast cancer death. These measures are imperfect, since 30% of the patients who should have a good outcome (no cancer spread to local lymph nodes, or "node-negative breast cancer"), eventually recur and die of breast cancer. Because breast cancer metastasis is so hard to predict, and so deadly, most low-risk node-negative breast cancer patients receive the same drug therapies routinely given to high-risk node-positive patients. This means that the majority of the low-risk node-negative breast cancer patients receive aggressive treatment they do not need. Our objective is to identify biomarkers that better define the metastatic potential of a node-negative breast cancer. We hypothesize that patterns of gene expression exist that distinguish primary breast cancers at low versus high risk of metastatic spread, and that these patterns can be ascertained using cDNA expression array technology, comparing frozen primary breast cancers of known good versus bad outcome. Multivariate analyses between these genes and with existing prognostic factors will determine the value of this approach in selecting optimal treatment strategies for women with node-negative breast cancer. With this information, clinicians could identify node-negative patients who require additional drug therapy for their disease, and could avoid over-treating those patients with very low risk of metastatic disease				
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Introduction

The presence or absence of systemic disease is the most crucial factor in survival versus mortality in women with breast cancer. Identifying high risk women and ensuring they receive appropriate adjuvant chemotherapy reduces risk of death from breast cancer metastasis. However, other than local cancer spread to lymph nodes and tumor size, few clinically useful prognostic markers exist, especially for lymph negative patients.

We therefore set out to identify gene expression patterns in primary breast cancer specimens that might dichotomize longer-term risk of recurrence, and stratify risk. The purpose of this study was to (1) Demonstrate that sufficient mRNA could be obtained from core biopsies to access gene expression, (2) Identify groups of genes that could be used to distinguish primary breast cancers with high recurrence risk, and (3) Validate genes that herald risks for systemic disease. At the time we began this study, we proposed to use frozen primary tumor tissues (with clinical follow-up) and commercially available "macroarrays" (Atlas spotted cDNA arrays). Two unforeseen setbacks complicated conduct of this set of experiments: Tests of 25 tumor samples indicated that the Atlas macroarray format was suboptimal to for rapid discovery of new prognostic markers of risk of systemic breast cancer; and 2) TS Allision flooded the Texas Medical Center and destroyed alternative specimens that might have used to pursue these studies using an alternative array format.

We modified our goals to adjust to these setbacks while continuing to address the aims proposed in the application. Since we had hypothesized that differential gene expression can stratify risk, we choose to forgo the gene expression analysis and focus on a candidate gene, metastasis-associated 1 (MTA1) that had been implicated both by genomic analysis and by the preliminary Atlas array analysis.

Body of Research

MTA1 functions in the cell nucleus as a steroid hormone receptor co-repressor (2), but inferring a specific role for MTA1 in metastasis is complicated by the rapidly growing MTA-gene family's at least six alternatively spliced forms encoded at three separate loci (i.e., MTA1 at 14q; MTA2 (aka MTA1L1) at -11q; MTA3 at 2q). Multiple alignment of MTA1, MTA2, and MTA3 gene open reading frames identified an MTA1-specific peptide that attached to a hapten, permitted us to generate a rabbit anti-MTA1 polyclonal antibody. MTA1 undergoes alternative splicing to both full length MTA1 and a recently described "short" cytoplasmic isoform (MTA1s), which shares full length MTA1's N-terminus, but replaces the C-terminal SH2-nuclear localization domain with a distinct C-terminal ER-binding (LRILL) motif (2). MTA1s interacts with ER α in cytoplasm rather than the nucleus (2). More recently, our data suggest that numerous additional alternatively spliced forms MTA1 exist.

To clinically validate MTA1 as a prognostic marker for breast cancer metastasis, we studied a large collection of archival primary breast cancers (salvaged from the flooded tumor bank) with an average of 8.8 years of clinical follow-up. Only 15% of the primary breast tumours studied showed significant cytoplasmic immunohistochemistry (IHC) staining, so to avoid confusion based on MTA1s' likely alternative function, only nuclear IHC signals were scored for these analyses. MTA1 nuclear IHC signals were scored on a range of 0-8 by adding a five point proportional score for percent of IHC positive cells to a three point IHC staining intensity scale (3). To define MTA1 overexpression, we compared MTA1 nuclear IHC scores measured in normal versus tumour tissues. Breast tumour specimens tested had a significantly higher IHC score (3.57 versus 5.07, respectively, for normal and tumour tissues; $p < 0.0002$). As IHC scores exceeding 5 occurred in less than 5% of normal tissues, we defined MTA1 overexpression as an IHC score equal to or greater than 6. Correlation analyses found no association between MTA1 expression, positive lymph nodes, or tumour size. Multivariate analysis of the full tumour set

revealed that MTA1 overexpression was significantly associated with early relapse (HR = 1.91 p = 0.0015). To avoid bias created by adjuvant endocrine and/or cytotoxic therapies, node-negative patients were separated into treated (N=217) and untreated (N= 397) subsets. As shown in Table 1, in the untreated patient subset, both univariate and multivariate analysis indicated MTA1 overexpression was a strong prognostic indicator of early disease recurrence (HR = 2.68, p = 0.0006), outperforming both tumour size (HR = 1.41, p = 0.039), and S-phase fraction (HR = 1.26, p = 0.072).

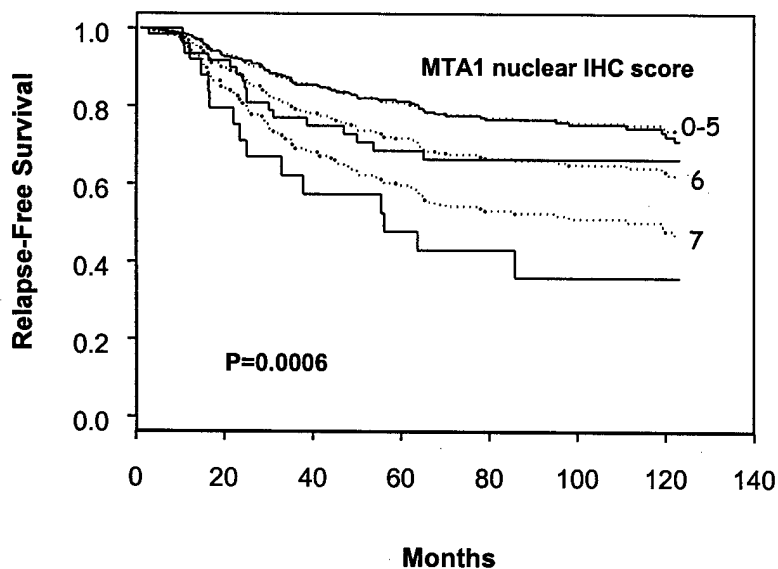
Table 1. MTA1 levels Relapse-free and Overall Survival in Node-Negative Patients

Variable	P Value	Hazard Ratio ¹	P Value	HazardRatio ¹
Untreated (N=394) 102 recurrence 138 deaths				
MTA1_cut6	0.052	2.68 (1.53-4.69)		
Tumor Size	0.17	1.26 (0.99-1.60)		
S-phase fraction	0.0012	1.41 (1.02-1.95)	0.046	1.32 (1.01-1.74)
Treated (N=217) 33 recurrences 34 deaths				
MTA1_cut6	0.61			
S-phase Fraction	0.039	1.58 (1.02-2.42)		
Tumor Size	0.78			

As indicated in **Figure 1**, the 23% (93/394) of untreated node-negative patients whose tumours overexpressed MTA1 levels had significantly increased risk of early disease (p= 0.0001 in univariate analysis and p = 0.0006 in multivariate analysis). For the 7% (29/394) patients whose tumours expressed the highest levels of MTA1 (IHC score 7-8), relapse rates exceeded 60%. **Table 1** indicates that despite MTA1's nearly 2-fold increase in recurrence risk, neither univariate nor multivariate models of overall survival detected any association between MTA1 overexpression and earlier patient death (p = 0.42). **Table 1** also shows that the treated subset of node-negative patients had no MTA1-associated increase in recurrence risk (p = 0.61). Given that all untreated node-negative patients who recurred received adjuvant therapy [I don't

understand; how could untreated patients have received adjuvant therapy?], this observation suggests that MTA1 overexpression is associated with enhanced treatment response.

Figure 1. Kaplan-Meier estimates (solid lines) and corresponding Cox regression estimates (dotted lines) are shown for various values of MTA1 in node-negative untreated subjects (N=397). MTA1 values of 0-5, 6, 7, and 8 were coded as 0, 1, 2, and 3 respectively in the analysis. Kaplan-Meier estimates and log rank tests were used to display and test the univariate association between RFS or OS and MTA1. Cox proportional hazards regression was used to test the independent contribution of MTA1 after accounting for other potentially important covariates. Adjusted survival curves were generated using Cox regression estimates for various values of MTA1, with cohort averages being used for other covariates in the model. Plots have been truncated at 120 months for graphical presentation, but all data were included in the analyses. Analyses were performed using the SAS (Version 8.2, Cary NC), and Splus (Version 6.1, Insightful, Seattle, WA).



These findings suggest MTA1 overexpression is an independent prognostic indicator of risk of early relapse, especially in untreated lymph node-negative primary breast cancers. MTA1 overexpression fails to directly associate with robust indicators of recurrence such as tumor size and lymph node status, suggesting that MTA1-facilitated distant spread is independent of, and perhaps distinct from, node-positive, disease-associated metastasis. As a result, measurement of MTA1 by IHC gleaned independent information and increased the sensitivity of multivariate models of prognosis. MTA1 retained its prognostic significance in node-negative disease, and

dichotomized otherwise unremarkable untreated node-negative primary breast tumors in low versus high risk subsets. Surprising as MTA1 overexpression-associated endocrine and cytotoxic tumour cross-sensitivity to treatment might appear, in an essentially unrelated study that considered neoadjuvant docetaxel response as a function of pre-treatment breast tumour gene expression profiles, we found MTA1 mRNA levels were elevated 2.9-fold ($p = 0.0085$) in the docetaxel-sensitive primary tumors (4).

Key Research Accomplishments

Two abstracts were accepted for to the San Antonio Breast Cancer Symposium in 2001-2002. A manuscript was published in Cancer Research, and a second to the Journal of the National Cancer Institute, which was returned based on reviewer's comments, revised and submitted to Lancet, but returned without review based on an editorial decision. The Lancet version of this manuscript is attached, and it will be reformatted and resubmitted to a "to be determined" journal this month.

Reportable Outcomes

1. J Chang, P O'Connell, S. Hilsenbeck. Feasibility of measuring gene expression using core biopsies of human primary breast cancers and cDNA microarray technology. Breast Cancer Res and Treat (suppl) 2000.
2. Martin MD, Fischbach K, Osborne CK, Mohsin SK, Allred DC, O'Connell P. Loss of heterozygosity events impeding breast cancer metastasis contain the MTA1 gene. Cancer Res 61:3578-3580, 2001

3. Genetic markers for response to neoadjuvant therapy: Array based gene expression profiling from serial biopsies. EC Wooten, J Chang, SG Hilsenbeck. 24th Annual San Antonio Breast Cancer Symposium, San Antonio, Texas (abstract 236), December 2001.

4. Martin MD, Hilsenbeck S, Mohsin SK, Hopp, TA, Clark GM, Osborne CK , Allred DC, O'Connell P. Breast tumours overexpressing nuclear isoforms of metastasis-associated 1 (MTA1) protein have high recurrence risks and enhanced response to systemic therapies (to be resubmitted)

Conclusion

While unforeseen difficulties prevented execution of the proposed studies as laid out in the proposal, significant progress on markers of breast cancer metastasis and the clinical relevance of the MTA1 protein in systemic disease resulted from the DAMD17-01-1-0478 study entitled "Can Gene Expression Pattern Analysis Predict Recurrence in Node-Negative Breast Cancer?"

In summary, our data suggest that measuring MTA1 protein expression in primary breast tumors identifies a high-risk subset of node-negative patients who need aggressive treatment, and a larger subset with no MTA1-associated recurrence risks. Furthermore, the strong association between MTA1 overexpression and enhanced treatment response has potential implications for all breast cancer patients, and warrants additional study.

1. A MTA1-specific antisera specific to the 14q32 gene locus
2. Abstracts and publications as noted above
3. Award of a follow-up concept grant exploring MTA1- regulated gene expression
4. New award of a "V"-Foundation clinical translational award (PI: O'Connell, P, effective 10/04) to examine how MTA1 isoform expression affects gene expression-based

predictive factor assays.

Please accept my thanks to the both the DoD Breast Cancer Research Program and the U.S. Army Medical Research and Materiel Command for their support of my research.

APPENDIX

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Breast tumours that overexpress nuclear metastasis-associated 1 (MTA1) protein have high recurrence risks but enhanced responses to systemic therapies.

Michelle D. Martin^{1,2}, Susan G. Hilsenbeck^{2,3}, Syed K. Mohsin^{2,4}, Torsten A. Hopp^{2,3}, Gary M. Clark^{2,3}, C. Kent Osborne^{2,3}, D. Craig Allred^{2,4}, and Peter O'Connell^{1,2,5*}.

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¹Department of Molecular and Cellular Biology, ²Breast Center, ³Department of Medicine, ⁴Department of Pathology, Baylor College of Medicine, Houston, Texas 77030, and ⁵Department of Human Genetics, Virginia Commonwealth University, Richmond, Virginia 23298-0033.

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*Correspondence should be addressed to:

Peter O'Connell, PhD, Department of Human Genetics, VCU School of Medicine, 1101 East Marshall Street, P.O. Box 980033, Richmond, Virginia 23298-0033

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Deleted: Virginia Commonwealth University

Tel: (804) 828-9632, X240

Fax: (804) 828-3760

E-mail: poconnell@vcu.edu

Requests for reprints should be addressed to: Peter O'Connell, Ph.D., Department of Human Genetics, Virginia Commonwealth University, P.O. Box 980033, Richmond, Virginia 23298-0033.

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Report (856 words)

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Nuclear metastasis-associated one (MTA1) protein is an oestrogen receptor co-repressor regulating transcription via chromatin remodeling. MTA1 mRNA levels are elevated in metastatic relative to non-metastatic tumours. MTA1 loss of heterozygosity is significantly less frequent in node-positive relative to node-negative breast tumours, suggesting epigenetic alterations of MTA1 affect metastatic potential (1)

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Immunohistochemistry showed that MTA1 overexpressing tumours have recurrence

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risks similar to node-positive tumours. Untreated node-negative tumours that

overexpressed MTA1 had the highest relapse risk (HR = 2.68, p = 0.0006).

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Chemotherapy eliminated all MTA1 associations with clinical outcome, suggesting

MTA1 overexpression predicts early relapse, but is associated with enhanced

chemoresponse.

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The prognostic significance of lymph node metastases has long been known to dichotomize risk of local versus systemic breast cancer, as have steroid hormone receptors' predictive value in selection of adjuvant hormonal therapy versus cytotoxic chemotherapies. While extremely useful, the risk factors in current use have limits. Finding a breast tumour to be oestrogen receptor-negative cannot infer the patient's optimal chemotherapy regimen, and the absence of lymph node metastases cannot stratify relapse risks for node-negative patients. Due to improved awareness and screening programs, women with primary breast cancer increasingly present with node-negative disease. Since the biomarkers in current use cannot differentiate risks for node-negative patients, most opt for chemotherapy, although relatively few stand to benefit. Effective prognostic indicators of micrometastasis could stratify recurrence risks and adjuvant therapy benefits, sparing the majority of these women from the toxicity and cost of chemotherapy.

MTA1 is a steroid hormone receptor co-repressor (2), but inferring a specific role for MTA1 in metastasis is complicated by the rapidly growing MTA-gene family's at least six alternatively spliced forms encoded at three separate loci (i.e., MTA1 at 14q; MTA2 at 11q; MTA3 at 2q). Multiple alignment of MTA gene family open reading frames identified an MTA1-specific peptide that when attached to a hapten, generated a rabbit anti-MTA1 polyclonal antibody. MTA1 undergoes alternative splicing to both full length MTA1 and a previously described "short" cytoplasmic isoform (MTA1s) that replaces full length MTA1's C-terminal src homology and nuclear localization domains with a distinct C-terminal ER-binding (LRILL) motif (2). MTA1s interacts with ER α in cytoplasm rather than the nucleus (2).

We studied a large collection of archived primary breast cancers with an average of 8.8 years of clinical follow-up. Only 15% of the primary breast tumours studied showed significant cytoplasmic immunohistochemical (IHC) staining, but to avoid confusion based on MTA1s' likely alternative function, only nuclear IHC signals were scored for these analyses. MTA1 nuclear IHC signals were scored on a range of 0-8 by adding a five point proportional score for percent of IHC positive cells to a three point IHC staining intensity scale (3). To define MTA1 overexpression, we compared MTA1 nuclear IHC scores measured in normal versus tumour tissues. Breast tumour specimens tested had a significantly higher

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In summary, our data suggest that measuring MTA1 protein expression in primary breast tumours identifies a high-risk subset of node-negative patients who need aggressive treatment, and a larger subset with no MTA1-associated recurrence risks. Furthermore, the strong association between MTA1 overexpression and enhanced treatment response has potential implications for all breast cancer patients, and warrants additional study.

Contributors

Experimental work was done by M.D. Martin. T.A. Hopp assisted M.D. Martin with characterization of the MTA1 antibody. Statistical analyses were done by G.M. Clark and S.G. Hilsenbeck. S.K. Mohsin and D.C. Allred assessed tumor pathology and constructed tissue arrays, and supervised assessment of MTA1 immunohistochemistry. C.K. Osborne, G.M. Clark, and D.C. Allred assembled the breast tumor specimens, and patient outcome data used in the study. M.D. Martin and P. O'Connell conducted the experimental studies described herein and authored successive drafts of the manuscript. D.C. Allred and P. O'Connell designed and supervised the overall study.

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Conflict of Interest Statement

M.D. Martin, G.M. Clark, D.C. Allred, and P. O'Connell, filed a US patent for measurement of MTA1 expression in breast cancer.

Acknowledgements

The authors thank Drs Gary Chamness and Jenny Chang for critical reading of the manuscript. This report was supported by grants CA30195 and CA58183 from the National Cancer Institute, National Institutes of Health, Department of Human Services, and USAMRC Breast Cancer Training Grant, DAMD-

M. Martin et al.

17-99-1-9047873. This study was conducted under the aegis of Baylor College of Medicine Institutional

Review Board, Protocol 47830.

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Figure 1. Kaplan-Meier Curves. Estimates of relapse-free survival and corresponding Cox regression

estimates are shown for various values of MTA1 in node-negative untreated subjects (N=397). Solid

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lines represent Kaplan-Meier curves, and dashed lines represent Cox regression estimates. MTA1 values

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of 0-5, 6, 7, and 8 were coded as 0, 1, 2, and 3 respectively in the analysis. Kaplan-Meier estimates and

log rank tests were used to display and test the univariate association between RFS or OS and MTA1.

Cox proportional hazards regression was used to test the independent contribution of MTA1 after

accounting for other potentially important covariates. Adjusted survival curves were generated using Cox

regression estimates for various values of MTA1, with cohort averages being used for other covariates in

the model. Plots have been truncated at 120 months for graphical presentation, but all data were included

in the analyses. Analyses were performed using the SAS (Version 8.2, Cary NC), and Splus (Version 6.1,

Insightful, Seattle, WA).

(for figure, refer to figure 1 on page 5 of this report)